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Inhibition of lipoprotein-associated phospholipase A_2 diminishes the death-inducing effects of oxidised LDL on human monocyte-macrophages

Keri L.H. Carpenter[a,*], Ian F. Dennis[a], Iain R. Challis[a], David P. Osborn[b], Colin H. Macphee[c], David S. Leake[d], Mark J. Arends[a], Malcolm J. Mitchinson[a]

[a]*Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK*
[b]*University Chemical Laboratory, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK*
[c]*Department of Vascular Biology, Glaxo SmithKline Pharmaceuticals, New Frontiers Science Park North, Third Avenue, The Pinnacles, Harlow CM19 5AW, UK*
[d]*Cell and Molecular Biology Research Division, School of Animal and Microbial Sciences, The University of Reading, Whiteknights, P.O. Box 228, Reading RG6 6AJ, UK*

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Abstract **The death of macrophages contributes to atheroma formation. Oxidation renders low-density lipoprotein (LDL) cytotoxic to human monocyte-macrophages. Lipoprotein-associated phospholipase A_2 (Lp-PLA_2), also termed platelet-activating factor acetylhydrolase, hydrolyses oxidised phospholipids. Inhibition of Lp-PLA_2 by diisopropyl fluorophosphate or Pefabloc (broad-spectrum serine esterase/protease inhibitors), or SB222657 (a specific inhibitor of Lp-PLA_2) did not prevent LDL oxidation, but diminished the ensuing toxicity and apoptosis induction when the LDL was oxidised, and inhibited the rise in lysophosphatidylcholine levels that occurred in the inhibitors' absence. Hydrolysis products of oxidised phospholipids thus account for over a third of the cytotoxic and apoptosis-inducing effects of oxidised LDL on macrophages.** © **2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Toxicity; Oxidized low-density lipoprotein; Lipoprotein-associated phospholipase A_2; Lysophosphatidylcholine; Oxidized phospholipid; Monocyte-macrophage (human)

*Corresponding author. Fax: (44)-1223-333720.
E-mail address: klhc@mole.bio.cam.ac.uk (K.L.H. Carpenter).

Abbreviations: CECA, cholesterol ester-derived core aldehyde; DCM, dichloromethane; DFP, diisopropyl fluorophosphate; DMS, dimethylsulphide; DMSO, dimethylsulphoxide; DNPH, 2,4-dinitrophenylhydrazone; ELISA, enzyme-linked immunosorbent assay; GC, gas chromatography; HDL, high-density lipoprotein; HMM, human monocyte-macrophage; HNE, 4-hydroxynon-2-enal; HPLC, high pressure liquid chromatography; 7β-HC, 7β-hydroxycholesterol; IN-oxLDL, inhibitor-pretreated oxidised low-density lipoprotein; IPA, isopropanol; LDH, lactate dehydrogenase; LDL, low-density lipoprotein; Lp-PLA_2, lipoprotein-associated phospholipase A_2; lyso-PC, lysophosphatidylcholine; M/DAG, mono- and diacylglycerol; oxLDL, oxidised low-density lipoprotein; oxNEFA, oxidised non-esterified fatty acid; PAF, platelet-activating factor; PAF-AH, platelet-activating factor acetylhydrolase; PC, phosphatidylcholine; PC-9-oxo, 1-palmitoyl-2-(9-oxononanoyl)-*sn*-glycero-3-phosphocholine; PCCA, PC-derived core aldehyde; REM, relative electrophoretic mobility; SMC, *smooth muscle cell*; TBARS, *thiobarbituric acid-reactive substances*; TLC, thin layer chromatography

1. Introduction

Amongst the evidence implicating low-density lipoprotein (LDL) oxidation in atherosclerosis progression is that oxidised LDL (oxLDL) has toxic and apoptosis-inducing effects on macrophages and smooth muscle cells (SMCs) [1–5]. Some of these cells die, by apoptosis and necrosis, in advanced atherosclerotic lesions [6,7]. Death of macrophage foam cells leads to lipid spillage and development of the lipid core (atheroma) of advanced plaques [8], whilst death of SMCs attenuates the fibrous cap. Such changes destabilise lesions, predisposing them to rupture, leading to thrombosis [9–11].

OxLDL contains a complex, incompletely characterised mixture of toxic oxidation products, including oxysterols, lipid hydroperoxides and their breakdown products, e.g. 4-hydroxynon-2-enal (HNE) [12–15]. Also produced are the core aldehydes, of unknown toxicity, in which an unsaturated fatty acid chain (originally usually 18 or 20 carbons) has become peroxidised and oxidatively fragmented (typically to nine or five carbons, terminating in an aldehyde group) but retains its ester link to the parent lipid. Phosphatidylcholine (PC)-derived core aldehydes (PCCAs) were first described as oxidatively fragmented PCs [16]. PCCAs and cholesterol ester-derived core aldehydes (CECAs) occur in Cu^{2+}-oxLDL [17,18]. CECAs were found in LDL oxidised by J774 macrophage-like murine cells [19] and in human atherosclerotic lesions [20,21].

Oxidised PCs, including PCCAs, have platelet-activating factor (PAF)-like pro-inflammatory activity [22,23] and are mitogenic for SMCs [23]. PCCAs are substrates for lipoprotein-associated phospholipase A_2 (Lp-PLA_2), an enzyme associated with LDL, also known as platelet-activating factor acetylhydrolase (PAF-AH). Lp-PLA_2 hydrolyses PCCAs to lysophosphatidylcholine (lyso-PC), whilst non-oxidised PCs are insusceptible [16,24].

We therefore investigated the role of LDL-associated Lp-PLA_2 in the cytotoxicity and apoptosis-inducing effects of oxLDL for human monocyte-macrophages (HMMs) in vitro.

2. Materials and methods

Pefabloc (4-(2-aminoethyl)-benzenesulphonyl fluoride) and the lac-

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tate dehydrogenase (LDH) release assay kit were from Roche (Lewes, UK). Diisopropyl fluorophosphate (DFP) was from Aldrich (Gillingham, UK). SB222657 was a gift of Glaxo SmithKline (Harlow, UK). The nucleosome enzyme-linked immunosorbent assay (ELISA) kit was from Oncogene (CN Biosciences, Nottingham, UK). Other chemicals, culture plates and media were as described previously [3].

The isolation of human monocytes from blood (normal volunteers) or buffy coat (National Blood Service), HMM culture (in Macrophage-SFM serum-free medium), LDL preparation, HMM-mediated LDL oxidation (in Ham's F-10 medium supplemented with 3 μM Fe_2SO_4, 10.8 mg/l phenol red and 2 mM glutamine), measurement of relative electrophoretic mobility (REM) and of thiobarbituric acid-reactive substances (TBARS) was as described previously [1,3,25,26]. Measurement by gas chromatography (GC) of free plus esterified individual fatty acids and 7β-hydroxycholesterol (7β-HC) in LDL and oxLDL was as before [3]. HMMs were cultured in Macrophage-SFM for 1–2 days prior to starting experiments.

1-Palmitoyl-2-(9-oxononanoyl)-*sn*-glycero-3-phosphocholine (PC-9-oxo) was synthesised by bubbling ozone from a Pennwalt ozone generator (100–140 V; 50 ml/min) through a solution of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (100 mg; Sigma) in dichloromethane (DCM; 40 ml) at −70°C for c. 1 h until it turned blue (excess ozone) indicating completion. Excess ozone was purged out (3 min) using oxygen; dimethylsulphide (DMS, 0.5 ml) was added (−70°C; 1 h), and then allowed to warm to room temperature. DCM and DMS were removed by rotary evaporation, then by a vacuum line. PC-9-oxo was purified by column chromatography on alumina (Brockman grade II–III), eluting with an increasing gradient of methanol in chloroform. The principle of ozonolysis was employed previously for PCCA synthesis [16,27,28]. CECAs were synthesised from cholesterol esters using osmium tetroxide by the method of Kamido et al. [36] and by our ozonolysis procedure (similar but not identical to that above). Molar yields of aldehydes synthesised by ozonolysis were c. 50%. Products were characterised by ^1H NMR (600 MHz), fast atom bombardment mass spectrometry, thin layer chromatography (TLC) and reversed-phase high pressure liquid chromatography (HPLC).

Liposomes were made by injecting solutions (40–100 mM; in ethanol) of PC-9-oxo, or non-oxidised PC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), or lyso-PC (1-hexadecanoyl-*sn*-glycero-3-phosphocholine; Sigma) through a fine needle into rapidly stirred incubation medium (Macrophage-SFM) at 37°C to give the desired final concentrations [29]. The ethanol vehicle (not exceeding 1% v/v) was non-toxic to the HMM cultures.

To irreversibly inhibit Lp-PLA_2, LDL (3 mg LDL protein/ml) was pretreated for 5 h at 37°C with 10 mM DFP [30] using isopropanol (IPA; 0.5% v/v) as the solvent vehicle. Excess DFP and IPA vehicle were then removed by dialysis (overnight with three buffer changes) against phosphate-buffered saline (PBS). Control LDL was prepared in parallel, omitting the DFP but with IPA and all other conditions identical. Pefabloc [31,40] and SB222657 [32] were also used to inhibit Lp-PLA_2. LDL (1 mg LDL protein/ml) was pretreated with 500 μM Pefabloc (water-soluble) or 2 μM SB222657 (vehicle dimethylsulphoxide (DMSO); 0.5% v/v) for 30 min at 37°C; control LDL (no inhibitor) was maintained in parallel. The LDLs were diluted to 1 mg LDL protein/ml in PBS, and were oxidised by exposure to copper sulphate (10 μM) for 15 h at 37°C. Cu^{2+} was removed by Chelex-100 resin [26], and the oxLDLs were added to HMM cultures in Macrophage-SFM at 200 μg LDL protein/ml. The final concentrations of Pefabloc or SB222657 were 100 μM or 0.4 μM, respectively. Incubations were up to 54 h. Toxicity was assessed by [^{3}H]adenine release (HMMs preloaded with [^{3}H]adenine; 0.25 μCi per well) [1] and by LDH release. Apoptosis was measured by nucleosome ELISA. Pefabloc and SB222657 (no LDL) were themselves non-toxic to HMMs at the final concentrations used. The DMSO vehicle used as above was non-toxic to HMMs and did not affect LDL oxidation. SB222657 (no LDL) gave background levels of apoptosis similar to no additions HMMs. DFP (10 mM) employed as above inhibited 97% of the native LDL-associated Lp-PLA_2 activity, using a published method to measure the hydrolysis of [^{3}H]PAF (NEN, Boston, MA, USA) to [^{3}H]lyso-PAF [33]. Cu^{2+}-mediated oxidation of LDL (DFP-pretreated and its control as above) was also performed at 0.2 mg LDL protein/ml in PBS using 5 μM Cu^{2+} for up to 72 h at 37°C, then stopped using EDTA (1.6 mM) and butylated hydroxytoluene (32 μM) and stored at −20°C until analysis.

For HMM-mediated LDL oxidation, native LDL was pretreated with DFP as above. Pretreatment of native LDL with Pefabloc (500 μM) was at 2 mg LDL protein/ml in PBS containing 27 μM EDTA, for 30 min at 37°C. Pretreatment of native LDL with SB222657 (2 μM) was at 200 μg LDL protein/ml for 30 min at 37°C in Ham's F-10 medium. The pretreated native LDLs were then added (individually) to HMM cultures in Ham's F-10 medium to give final concentrations of 200 μg LDL protein/ml. Toxicity was monitored for up to 72 h by the [^{3}H]adenine release method [3].

For aldehyde analysis, 4 ml of oxLDL (200 μg LDL protein/ml oxidised by 5 μM Cu^{2+} or by HMMs) were mixed with 4 ml 2,4-dinitrophenylhydrazine solution (1.77 mM in 1 M HCl), to form the 2,4-dinitrophenylhydrazone (DNPH) derivatives of the aldehydes [34,35]. After 2 h in the dark at room temperature, the aldehyde-DNPH derivatives were extracted [35] and separated on silica gel TLC plates [35], eluted as described [18]. Bands were scraped and extracted, using DCM for hexanal and CECAs, DCM/methanol (2:1, v/v) for HNE, and DCM/methanol (1:1, v/v) for PCCAs. The extracts were evaporated under a stream of nitrogen, redissolved in methanol and injected onto a reversed-phase column (Spherisorb ODS2, 4.6 mm×25 cm; Waters; 40°C) on a Hewlett Packard 1050 HPLC. The mobile phase for PCCAs was isocratic methanol/water/acetonitrile 93:7:5 (v/v/v) containing 20 mM choline chloride [16], 1 ml/min, detected at 378 nm. Mobile phases and detection wavelengths for HNE and CECAs were as described previously [18,35]; those for hexanal were as for HNE. Components were identified by retention times and quantified by peak areas (using a Hewlett Packard ChemStation data system), compared with standards.

For analysis of lyso-PC and PC, samples of LDL and oxLDL (0.5 ml of 1 mg LDL protein/ml), to which an internal standard, 1,2-dipentadecanoyl-*sn*-glycero-3-phosphocholine (Sigma), was added (100 μl of a 1 mg/ml solution), were extracted by the Bligh and Dyer method and a phospholipid fraction isolated by the method of Kaluszny et al. [37] using aminopropyl Sep-pak cartridges (Millipore) on a Waters 24 position manifold. The phospholipids were dried down under N_2 and redissolved in diethyl ether (1 ml), then hydrolysed and analysed by our adaptation of published procedures [38], as follows. Phospholipase C (10–20 U/mg protein, type I from *Clostridium welchii*; Sigma P 7633) was dissolved at 5 mg/ml in buffer (50 mM Tris, 30 mM $CaCl_2$, pH 7.3) and 20 μl diluted with a further 1 ml of the same buffer. It was preextracted with 2×1 ml washes of diethyl ether (to remove lipid impurities), and then mixed with 1 ml of phospholipid solution (prepared above) and stirred overnight at room temperature. The diethyl ether layer, containing the liberated mono- and diacylglycerols (M/DAGs), was then collected, and pooled with a further 2×2 ml diethyl ether extracts of the aqueous phase, then dried under N_2. The M/DAGs were treated with bistrimethylsilyltrifluoroacetamide plus 1% trimethylchlorosilane (Pierce) to form trimethylsilylether (TMSi-ether) derivatives. Excess reagents were evaporated off under N_2. The M/DAG TMSi-ether derivatives were then dissolved in hexane (0.5 ml) and 1 μl analysed by GC (Carlo Erba GC8000). Injection was via a cold on-column injector onto a 30 m DB-1 column [3], temperature programmed 50–200°C at 10°C/min, 200–300°C at 4°C/min, and then held at 300°C for 20 min. The carrier gas was hydrogen and detection was by a flame ionisation detector. Quantitation was by peak areas measured by a Shimadzu CR3A integrator, relative to the internal standard.

Mean values of data are ± S.D. Where error bars are not visible in graphs, the S.D. is smaller than the relevant data point symbol. Statistical analysis was by Student's *t*-test (unpaired). Experiments were repeated several times and representative data are presented.

3. Results and discussion

All three inhibitors of Lp-PLA_2 – DFP, Pefabloc and SB222657 – resulted in diminished toxicity, determined by the [^{3}H]adenine release assay, when the inhibitor-pretreated LDL was subsequently oxidised by Cu^{2+} and then added to HMMs (Fig. 1a). The results for Pefabloc and SB222657 were confirmed by the LDH release assay (Fig. 1b). OxLDL-induced apoptosis in HMMs, measured by nucleosome ELISA, was similarly diminished by SB222657 (Fig. 1c).

Lyso-PC levels in control Cu^{2+}-oxLDL averaged 4.3-fold

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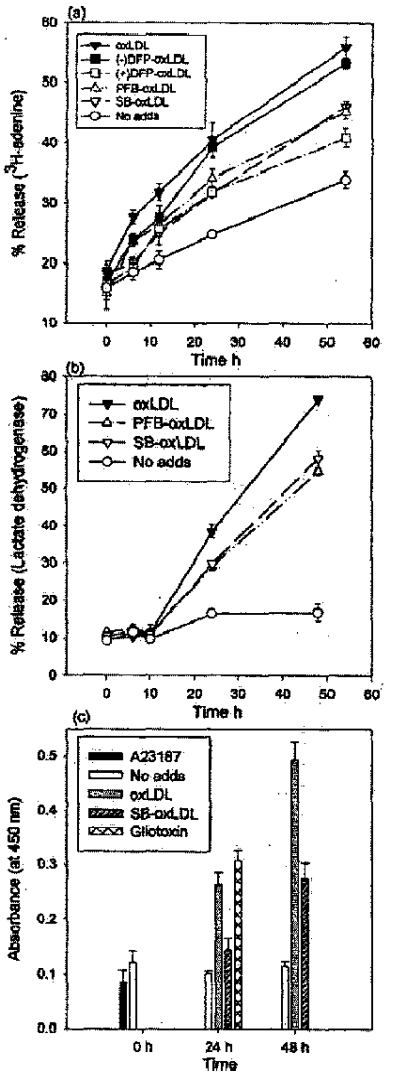
Fig. 1. Cytotoxicity to HMMs of Cu^{2+}-oxLDL, with or without pretreatment with inhibitors of Lp-PLA_2 prior to the oxidation. LDL was oxidised at 1 mg protein/ml in PBS, using 10 μM Cu^{2+} for 15 h at 37°C, then incubated with HMMs in Macrophage-SFM at 200 μg LDL protein/ml culture medium. For further details see Section 2. (a) Toxicity was measured by the [^{3}H]adenine release method. Data points are means of three to six replicate wells ± S.D. ▼, control oxLDL, no inhibitor; ■, control oxLDL, sham-pretreated with solvent vehicle as a control for DFP ((−)DFP-oxLDL); □, DFP (10 mM)-pretreated, oxLDL ((+)DFP-oxLDL); △, Pefabloc (500 μM)-pretreated oxLDL (PFB-oxLDL); ▽, SB222657 (2 μM)-pretreated oxLDL (SB-oxLDL); ○, no additions control (no adds), i.e. HMMs cultured in medium alone. Toxicity of inhibitor-pretreated oxLDL was significantly different from that of control oxLDL that had not been pretreated, or in the case of DFP, from that of control oxLDL that had been sham-pretreated with solvent vehicle, as follows: at 24 h and 54 h, for SB222657 $P<0.01$ and $P<0.0001$, respectively; for Pefabloc $P<0.05$ and $P<0.001$; for DFP $P<0.01$ and $P<0.001$. (b) Toxicity was measured by the LDH release method. Data points are means of triplicate wells ± S.D. Treatments and symbols are as in panel a except that DFP was not assessed. Toxicity of inhibitor-pretreated oxLDL was significantly different from that of control oxLDL, at 24 h and 48 h, respectively, for SB222657 $P<0.01$ and $P<0.001$; for Pefabloc $P<0.01$ and $P<0.0001$. (c) Apoptosis of HMMs was measured by nucleosome ELISA. Treatment was as in panel a for SB222657. DFP and Pefabloc were not assessed. Gliotoxin (5 μM) was a positive control for apoptosis. Calcium ionophore A23187 (100 μM; 0.5 h), a necrosis-inducing agent, was a negative control for apoptosis. Data are means of triplicate wells ± S.D. Apoptosis induced by SB-oxLDL was significantly different from that of control oxLDL, at 24 h and 48 h, respectively, $P<0.01$ and $P<0.001$.



higher than those of native LDL, but in those which had been pretreated with inhibitors of Lp-PLA_2 prior to oxidation (inhibitor-pretreated oxLDL, IN-oxLDL) lyso-PC only rose to 1.2–1.5-fold higher than native LDL (Fig. 2a). Inhibition of lyso-PC production was thus 85% or greater. PC loss as a result of oxidation was c. 40% regardless of inhibition of Lp-PLA_2 (Fig. 2b). Levels of PC cannot drop below c. 50% of native levels because the remainder possess saturated or mono-unsaturated chains that resist peroxidation [39]. The REMs of IN-oxLDL for all three inhibitors and control oxLDL were about equal (Fig. 2c). Loss of total free plus esterified linoleate was 59–67% and that of arachidonate was 86–92%, as a result of oxidation, regardless of inhibition of Lp-PLA_2 for all three inhibitors. 7β-HC was negligible in native LDL, whilst in IN-oxLDL (DFP-pretreated) and its corresponding control oxLDL 7β-HC levels were 30.6 and 25.3 μg/mg LDL protein, respectively. In IN-oxLDL (Pefabloc- or SB222657-pretreated) and control oxLDL, 7β-HC levels were 14.4, 11.3 and 12.9 μg/mg LDL protein, respectively. Levels of TBARS (nmol malondialdehyde/mg LDL protein) were not significantly different ($P>0.1$) for control oxLDL (40.6 ± 6.4, $n=5$) and IN-oxLDL (for Pefabloc, 43.6 ± 14.0, $n=5$; for SB222657 45.0 ± 8.2, $n=5$). Thus the diminished toxicity of IN-oxLDL relative to control oxLDL was not due to inhibition of oxidation per se.

Pretreatment of native LDL individually with DFP, Pefabloc or SB222657 conferred a variable degree of protection against the self-inflicted toxicity arising from HMM-mediated LDL oxidation, within the oxidising cultures themselves (in Ham's F-10 medium). This protection was only apparent in the later stages (> 24 h) and ranged from c. 30% inhibition with DFP to over 80% with SB222657 or Pefabloc.

PCCAs were undetectable in native LDL. After oxidation by Cu^{2+} for 20 h and 48 h, PCCA levels in IN-oxLDL (DFP-pretreated) were 6.8 and 10.4 nmol/mg LDL protein, respectively, whereas in corresponding control oxLDL they were 3.5 and 4.9 nmol/mg LDL protein. Corresponding REMs were 3.2 and 4.8 for IN-oxLDL and 3.0 and 4.7 for control oxLDL. Throughout a time course (0, 6, 12, 24, 48 and 72 h) production of HNE, hexanal and CECAs was similar in IN-oxLDL (DFP-pretreated) and control oxLDL. For example, hexanal levels peaked at 24 h, with 84.6 and 85.3 nmol/mg LDL protein for IN-oxLDL and control oxLDL, respectively. HNE also peaked at 24 h, with 27.5 and 26.1 nmol/mg LDL protein. Cholesteryl 9-oxononanoate peaked at 12 h, with 29.8 and 30.8 nmol/mg LDL protein. Cholesteryl 5-oxovalerate peaked at 6 h, with 6.0 and 6.3 nmol/mg LDL protein.

For HMM-mediated oxidation of LDL in Ham's F-10 medium, after 24, 48 and 72 h PCCA levels in IN-oxLDL (DFP-pretreated) were 7.2, 8.4 and 15.5 nmol/mg LDL protein, whilst for corresponding control oxLDL they were 0.9, 5.7 and 7.1 nmol/mg LDL protein. The corresponding REMs

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were 1.8, 2.6, and 3.0 for IN-oxLDL and 1.8, 2.4 and 2.9 for control oxLDL. CECAs were also detected in HMM-oxLDL, but the effect of inhibitors of Lp-PLA$_2$ on their production was not measured.

To assess the toxicities of lyso-PC and PCCAs, we added them individually (as liposomes) at a range of concentrations to cultures of HMMs in Macrophage-SFM. For lyso-PC, a sharp increase in toxicity was seen between 50 and 100 μM, and maximal toxicity was reached at 150 μM (Fig. 3a). By comparison the dose-related increase in toxicity of PC-9-oxo was gradual (Fig. 3b). Levels of lyso-PC in oxLDL, when translated into final concentrations in the culture medium, were 24–40 μM, i.e. slightly under the toxic threshold concentration for pure lyso-PC. Hydrolysis of oxidised PC theoretically yields 1 mol of oxidised non-esterified fatty acid (oxNEFA) per mol of lyso-PC liberated, so oxNEFA (presumably including aldehydic acids) might contribute to the toxicity.

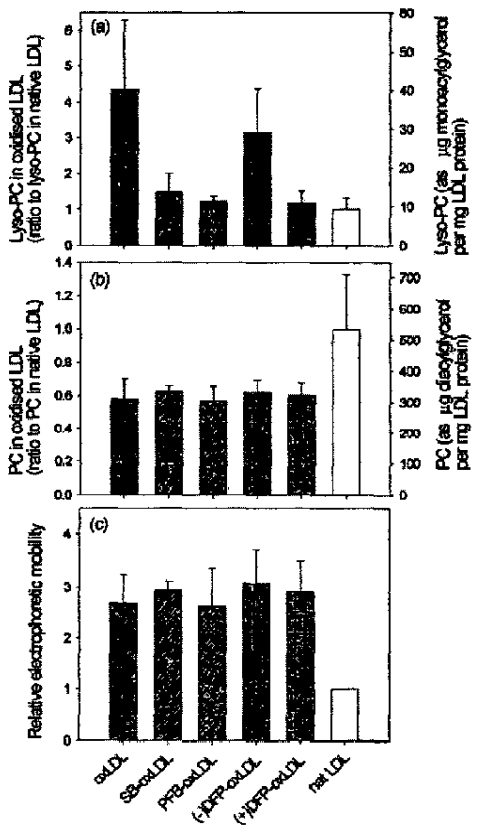

Fig. 2. Effect of inhibitors of Lp-PLA$_2$ on (a) levels of lyso-PC, (b) levels of PC and (c) REM for Cu^{2+}-oxLDL. Results are mean values (±S.D.) for native (nat) LDL ($n=6$), and for oxLDL ($n=5$), SB-oxLDL ($n=4$), PFB-oxLDL ($n=4$), (−)DFP-oxLDL ($n=4$) and (+)DFP-oxLDL ($n=4$), expressed relative to the corresponding native LDL in each case; n = number of experiments. Lyso-PC levels for SB-oxLDL, PFB-oxLDL and (+)DFP-oxLDL were significantly different ($P<0.03$) from those of oxLDL. Lyso-PC levels in (−)DFP-oxLDL were not significantly different from those of oxLDL ($P>0.1$). PC levels and REM for SB-oxLDL, PFB-oxLDL, (+)DFP-oxLDL and (−)DFP-oxLDL were not significantly different from those of oxLDL ($P>0.1$). Abbreviations and oxidation conditions are as in Fig. 1.

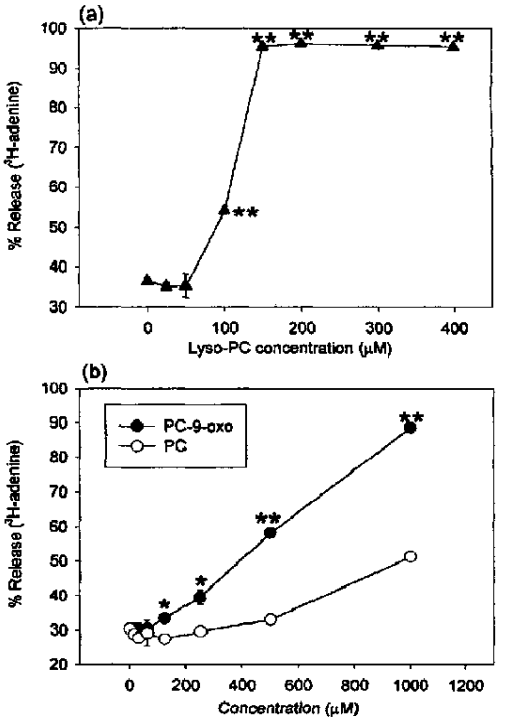

Fig. 3. Cytotoxicity to HMMs of (a) lyso-PC and (b) PC-9-oxo, measured by the [^{3}H]adenine release method. Incubations were for 24 h in Macrophage-SFM. Data points are means of triplicate wells ± S.D. *$P<0.01$, **$P<0.001$, toxicity significantly different (a) for lyso-PC compared with control (no lyso-PC) and (b) for PC-9-oxo compared with non-oxidised PC.

The combined effect of lyso-PC and oxNEFA with other cytotoxins in oxLDL, e.g. oxysterols, lipid hydroperoxides, and aldehydes such as HNE, may be important. PCCA levels in oxLDL corresponded to only a few μM final concentration in the medium, i.e. well below the toxic threshold concentration for synthetic PCCAs. Local concentrations of lyso-PC, oxNEFA or PCCAs within oxLDL particles would be higher than bulk concentrations in the medium, and this might affect toxicity.

The reduction in toxicity caused by the Lp-PLA$_2$ inhibitors is unlikely to be due to prevention of uptake of oxLDL by HMMs, as pretreatment of native LDL with DFP or SB222657 did not inhibit subsequent uptake by mouse peritoneal macrophages or J774 cells respectively, following oxidation by Cu^{2+} or mouse peritoneal macrophages ([30]; D.S. Leake and S.E. Stait, unpublished observations).

PCCA production during LDL oxidation only represents a few per cent of the loss of PC on a molar basis, even when Lp-PLA$_2$ is inhibited. Moreover, production of lyso-PC in control oxLDL is c. 30% (on a molar basis) of the loss of PC, suggesting that not all oxidised PC is hydrolysed, possibly because of rapid binding of oxidised PC (including PCCAs) to apolipoprotein B-100. Also PCCAs and lyso-PC might be further converted to other products not detectable by our assays. Exogenously added PCCAs bind covalently to proteins and peptides [41,42]. PCCAs and PCCA-modified protein are recognised by macrophages, and are epitopes involved

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in the antigenicity of oxLDL [28,43–45]. Lp-PLA_2 may be able to hydrolyse oxidised fatty acid chains in the *sn*-2 position of PC before they fragment into aldehydes [32], possibly explaining the low observed PCCA levels in control oxLDL.

The role of Lp-PLA_2 is vexed. Both lyso-PC and oxidatively fragmented phospholipids (including PCCAs) are mitogenic for SMCs [23,48] and appear involved in the antigenicity of oxLDL [28,43–45,49]. Oxidatively fragmented phospholipids possess pro-inflammatory, PAF-like activity [22,23], and as shown here, are cytotoxic to HMMs, albeit less so than lyso-PC. Lyso-PC is a chemoattractant for monocytes [47]. Lyso-PC is a detergent, and can, under some conditions, kill cells by disrupting the plasma membrane [50]. Lyso-PC can induce apoptosis in endothelial cells and SMCs in vitro [50,51], so might similarly affect macrophages. The oxNEFA fraction arising from Lp-PLA_2-mediated hydrolysis of oxLDL is a chemoattractant for monocytes, and pretreatment of LDL with SB222657 before oxidation inhibited this [32].

Plasma Lp-PLA_2 activity is predominantly associated with LDL, and a smaller proportion with high-density lipoprotein (HDL) [52–54]. HDL-associated Lp-PLA_2 may have a protective role in atherosclerosis [46,55]. The Lp-PLA_2 of LDL is predominantly associated with a small, dense atherogenic LDL sub-fraction [56]. Plasma Lp-PLA_2 activity appears partially heritable [57]. Lp-PLA_2 deficiency in plasma, due to a missense mutation, was a risk factor for ischaemic heart disease in Japanese men [58], but the overall significance of this mutation remains complex and unclear [59]. Deficiency in Lp-PLA_2 activity was reported in LDL from patients with familial hypercholesterolaemia [33]. Others have reported elevated plasma Lp-PLA_2 activity in patients with familial hypercholesterolaemia [60] and arterial disease [54,61,62]. Moreover, elevated levels of plasma Lp-PLA_2 were a strong independent predictor of coronary artery disease [63]. Lp-PLA_2 distribution between LDL and HDL, and LDL-associated Lp-PLA_2 activity in combination with oxidisability of LDL (influenced by dietary polyunsaturated fatty acids and antioxidants, e.g. vitamin E), might be important determinants of risk. Also the potentially relevant roles of intracellular and cell-secreted Lp-PLA_2/PAF-AH in tissues are little understood [64]. The toxicity of PC-9-oxo to HMMs in vitro (Fig. 3b) might arise at least partly from hydrolysis to lyso-PC and oxNEFA by Lp-PLA_2 secreted by the cells. In vitro, mature macrophages and, to a lesser degree, freshly isolated monocytes secrete Lp-PLA_2 identical to LDL-associated Lp-PLA_2 [65–67]. Lp-PLA_2 mRNA and protein occur in macrophages in human and rabbit atherosclerotic lesions [67].

The reduction in oxLDL-mediated death of HMMs, as a result of inhibition of Lp-PLA_2, was due at least partly to diminution of apoptosis, as judged by nucleosome ELISA. Supporting evidence from flow cytometry of propidium iodide-stained HMMs (following treatment with ethanol and RNase), using a published method [68], showed that 15% of total cells were hypodiploid (in a pattern typical of apoptotic cells) for uninhibited, oxLDL-treated HMMs (24 h), which decreased to 12% for IN-oxLDL (SB222657-pretreated), compared with a background of 3% in no additions control HMMs. Fluorescence microscopy of HMMs treated and stained as above revealed nuclear abnormalities characteristic of apoptosis (chromatin condensation or nuclear fragmentation) in c. 50%, 30% and 7% of the HMMs, respectively.

Although the details of the execution mechanism(s) are outside the scope of the present study, some important considerations follow. Internucleosomal cleavage of DNA has long been considered a hallmark of apoptosis [69,70] and is due to activation of caspase-activated DNase (CAD) as a result of caspase-mediated cleavage of its inhibitor, ICAD. Caspase activation (especially the executioner caspase 3) plays a pivotal role in apoptosis and caspase 3 activation was detected in Cu^{2+}-oxLDL-induced apoptosis in HMMs [68]. In human atherosclerotic lesions, colocalisation of CPP-32 (caspase 3) with TUNEL-positive cells (predominantly macrophages) suggests a role for caspase activation in apoptosis in atherosclerotic plaques in vivo [71], although both necrosis and apoptosis appear to occur in atherosclerotic plaques [7]. Classical apoptosis and necrosis have been suggested to represent the ends of a continuum of modes of death, with varying contributions of the cellular machinery [72]. Caspase-independent cell suicide can occur with certain combinations of cell types and stimuli, and other proteases may take over some of the roles of caspases [72]. Moreover, inhibiting caspase activation can reveal or even enhance caspase-independent cell death [72]. It was suggested that in atherosclerotic lesions macrophage necrosis might be promoted if their caspases are inactivated [7]. LDL oxidised by Cu^{2+} to a degree [26] similar to that used in the present study produced classical apoptotic nuclear ultrastructural changes in HMMs [1]. The effect of Lp-PLA_2 inhibition on caspase activation in oxLDL-treated HMMs is thus a potentially complex issue that merits investigation in its own right.

The role of Lp-PLA_2 in atherosclerosis is still incompletely understood. In this study, inhibition of Lp-PLA_2 typically diminished between a third and a half of the ensuing toxicity and apoptosis-inducing effect of the LDL when subsequently oxidised. The products of Lp-PLA_2-mediated hydrolysis of oxidised PC thus appear to account for a substantial proportion of the death of human macrophages. These findings may be relevant to the death of macrophage foam cells and SMCs in atherosclerotic lesions and progression of lesions to a state vulnerable to rupture.

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